Inhibition of Nucleotide Excision Repair by Fludarabine in Normal Lymphocytes *in vitro,* Measured by The Alkaline Single Cell Gel Electrophoresis (Comet) Assay

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F-ara-A Inhibits Nucleotide Excision Repair

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Summary

Alkylating agents or platinum analogues initiate several excision repair mechanisms, the processes of which include incision and excision of the damaged nucleotide, gap filling by DNA resynthesis, and rejoining by ligation. The previous study described that nucleotide excision repair permitted an incorporation of fludarabine nucleoside (F-ara-A) into the repair patch, thereby inhibiting the DNA resynthesis. In the present study, to clarify the repair kinetics in view of the inhibition by F-ara-A, normal lymphocytes were stimulated to undergo nucleotide excision repair by ultraviolet C (UV) in the presence or the absence of F-ara-A. The repair kinetics were determined as DNA single strand breaks resulting from the incision and the rejoining using the alkaline single cell gel electrophoresis (Comet) assay. DNA resynthesis was evaluated by the uptake of tritiated thymidine into DNA. The lymphocytes initiated the incision step maximally at 1 h, and completed the rejoining process within 4 h after UV exposure. UV also initiated the thymidine uptake, which increased time-dependently and reached the plateau at 4 h. A 2-h pre-incubation with F-ara-A inhibited the repair in a concentration-dependent manner, with the maximal inhibition by 5 μM. This inhibitory effect was demonstrated by the reduction

of the thymidine uptake and by the inhibition of the rejoining. A DNA polymerase inhibitor, aphidicolin, and a ribonucleotide reductase inhibitor, hydroxyurea, were not so inhibitory to the repair process as F-ara-A at equimolar concentrations. The present findings suggest that such a mechanistic interaction may be applicable for therapeutic strategy against cancer especially in the context of resistant cells with an increased repair capacity.

Introduction

Alkylating agents and platinum analogues are effective in the treatment of several kinds of hematological malignancies.[1-4] They exert their cytotoxicities by inducing DNA damage such as DNA adducts and DNA cross-links.[2, 5, 6] Cancer cells respond to these DNA insults by initiating several excision repair processes such as base excision repair, nucleotide excision repair, and recombinational repair.[7-9] The process of such excision repairs includes incision for removal of the damaged DNA, gap filling by DNA resynthesis, and rejoining by ligation.[10-13] If the repair process is inhibited, the cytotoxicity of these DNA damaging agents may be potentiated.[7-9]

The deoxyadenine nucleoside analogue monophosphate, 9-β-D-arabinofuranosyl-2-fluoroadenine-5'-monophosphate (fludarabine, F-ara-AMP) is currently used effectively for the treatment of leukemia and lymphoma.[14, 15] As the mechanism of action, the nucleoside form of fludarabine (9-β-D-arabinofuranosyl-2-fluoroadenine, F-ara-A) terminates DNA elongation after being incorporated into DNA.[16-19] It also inhibits ligation of DNA strands.[16-19] Moreover, the drug inhibits ribonucleotide reductase, causing a decrease in cellular

deoxyribonucleotide pools required for DNA synthesis.[20] Consequently, F-ara-A inhibits cellular DNA synthesis potently. The enzymes used in DNA replication are also involved in DNA repair. If so, F-ara-A may be capable of inhibiting DNA repair as it does replication.

The previous study described that nucleotide excision repair permitted an incorporation of F-ara-AMP into the repair patch.[21] This incorporation was associated with the inhibition of thymidine uptake into DNA, suggesting F-ara-A-mediated inhibition of the repair process. However, this previous study could not reveal the exact behavior of the repair kinetics in view of the inhibition by F-ara-A, because the evaluation of the thymidine uptake was insufficient for this purpose.

In the present study, we investigated the action of F-ara-A on the nucleotide excision repair process in normal lymphocytes *in vitro*. After the initiation of the repair, incision and rejoining in the repair process were determined as the amount of DNA single strand breaks using the alkaline single cell gel electrophoresis (Comet) assay.[22-24] Gap filling by DNA resynthesis was evaluated by the tritiated thymidine incorporation into DNA.[25] Quiescence of lymphocytes was used to prevent the

effect of F-ara-A on DNA replication during the cell cycle. Ultraviolet C (UV) was employed as a DNA-damaging modality because it induces a well-characterized nucleotide excision repair predominantly and have few effects on other macromolecules.[13] In addition, the inhibitory effect by F-ara-A was compared with those by a DNA polymerase inhibitor, aphidicolin, or a ribonucleotide reductase inhibitor, hydroxyurea, in the same experimental setting.[26, 27]

MATERIALS AND METHODS

Chemicals and reagents. F-ara-A, aphidicolin, and hydroxyurea were purchased from SIGMA (St. Louis, MO). [methyl, 1', 2'-^{3}H] thymidine (123 Ci/mmol) was purchased from Amersham International (Buckinghamshire, UK).

Lymphocyte preparation. Whole blood was drawn from 5 healthy donors into heparinized tubes, layered over Ficoll-Hipaque, and centrifuged at 1,500 rpm for 30 min.[28] The lymphocytes were harvested from the interphase, washed twice with PBS, and resuspended at 1 x 10^6 cells/ml in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Inc., Grand Island, NY) and 2 mM L-glutamine. The cells were incubated in a 5 % CO_2-humidified atmosphere at

37° C overnight.

UV exposure and drug treatments. The lymphocytes (1 x 10^6 cells/ml in 2 ml) were pre-incubated with F-ara-A at various concentrations for 2 h or not, followed by a 3 J/m^2 UV exposure. The cells were immediately followed by washing into fresh media and by incubating for the indicated periods.

The alkaline single cell gel electrophoresis (Comet) assay. To evaluate the DNA damage repair kinetics, the alkaline Comet assay was performed according to the method developed by Singh et al.[23] Approximately 5,000 lymphocytes after the treatment were mixed with 40 µl of 0.5% low melting point agarose in PBS at 37°C. The mixture was layered onto a frosted microscope slide previously coated with 100 µl of 0.65% normal agarose in PBS, followed by a top layer of 80 µl of the low melting point agarose. After solidification, the slide was left in the lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM ethylenediamine tetraacetic acid, 10% dimethylsulfoxide, 1% Triton X-100, pH 10) at 4°C for 1 h. The slide was then placed in the electrophoretic buffer (1 mM ethylenediamine tetraacetic acid, 300 mM NaOH, pH 13) for 20 min at 4°C to allow unwinding of DNA. Electrophoresis was conducted for the next 20 min at 0.7 V/cm and at 300 mA. After the electrophoresis, the slide was washed with the

neutralization buffer (0.4 M Tris, pH7.5) and stained with 25 µl of 20 µg/ml of ethidium bromide. One hundred cells per treatment were analyzed at 200 x magnification with an epifluorescence microscope (BX-50, OLYMPUS, Tokyo) connected through a black and white charge-coupled device video camera to the computer-based image analysis system (Kinetic Imaging Komet system, Ver. 4.0, Liverpool, UK). Cellular responses to DNA damage were expressed as the "tail moment", which combined a measurement of the length of the DNA migration and the relative DNA content therein.[29)]

Evaluation of DNA resynthesis. DNA resynthesis for gap filling during the repair was evaluated by the incorporation of tritiated thymidine into DNA.[21, 25)] After Normal lymphocytes (2 x 10^6 cells) had been exposed to UV with or without F-ara-A pre-treatment, the cells were washed into freash media, and incubated for the indicated time with tritiated thymidine (6 μCi). The lymphocytes were collected at 0 h, 1 h, 2 h, 4 h, and 6 h, centrifuged, and resuspended with 500 μl of 0.4 N perchloric acid. The samples were vortexed, centrifuged, and resuspended with perchloric acid again. After another vortex and centrifugation, the pellet was resuspended in 1 ml of

0.5 N KOH, and incubated at 45°C overnight to dissolve the pellets. The radioactivity was counted on the following day.

Quantitation of apoptotic cell death. To evaluate cytotoxicity, apoptotic cell death was determined by Hoechst staining at 24 h after the treatments. Cells having been treated and washed into fresh media were incubated with 2 μg/ml Hoechst No. 33342 for 30 min at 37 °C. Nuclei, 200 per treatment condition, were counted under UV illumination using a fluorescence microscope (BX-50, OLYMPUS, Tokyo). Apoptotic cell death was determined by the nuclear morphology with nuclear condensation and fragmentation.

Statistical analysis. All statistical analyses were performed with Microsoft Excel (Redmond, WA). All graphs, linear regression lines, and curves were generated with GraphPad Prism software (GraphPad Software, Inc. San Diego, CA).

RESULTS

Nucleotide excision repair initiated by UV. To determine the kinetics of UV-induced nucleotide excision repair, single strand breaks were measured by the Comet assay. When normal lymphocytes were irradiated, the tail moment was

greatest at 1 h after the exposure, suggesting the maximal DNA strand breaks resulting from the incision of the repair process (Figs. 1A and 3A). The tail moment decreased promptly thereafter, suggesting the rapid repair process enabling rejoining of the incised DNA. The tail-moment value came to the control level at 4 h, representing successful completion of the process (Figs. 1A and 3B). The tail moment values at 1 h became greater by the escalating doses of UV, suggesting that the single strand breaks were generated through the UV-induced repair (Fig. 1B).

Effect of F-ara-A on the DNA repair process. When the lymphocytes were pre-incubated for 2 h with 10 μM F-ara-A followed by UV exposure, the tail-moment value at 4 h was greater than the value generated by UV alone (Figs. 2 and 3D). The tail moment values at 1 h were not different between the presence or the absence of F-ara-A (Figs. 2 and 3C). Such an inhibitory effect was not obtained by 0.1 μM F-ara-A (Fig. 2).

Inhibition of the DNA resynthesis step. To reveal the action of F-ara-A on the DNA resynthesis step, the thymidine incorporation assay was performed. UV exposure initiated the incorporation of thymidine into DNA of lymphocytes, and this incorporation appeared to reach a plateau at 4 h, suggesting the completion of the

resynthesis (Fig. 4). Pre-treatment with 10 μM F-ara-A reduced the thymidine uptake from zero time, with a significant reduction at 6 h (P = 0.04 for paired samples).

Concentration-dependent inhibition of DNA repair by F-ara-A. To further evaluate F-ara-A-mediated inhibition of the repair, the lymphocytes were incubated with different concentrations of F-ara-A or not, followed by UV exposure and by washing into fresh media. The tail moment values at 4 h became greater concentration-dependently, suggesting the increased inhibitory effect on the rejoining step. This inhibition reached a maximum at 5 –10 μM F-ara-A (Fig. 5A). The thymidine uptake was also inhibited by the increasing concentrations of F-ara-A, with the effect peaking at 5 –10 μM (Fig. 5B).

Comparison of the inhibitory effects among aphidicolin, hydroxyurea, and F-ara-A. To compare the inhibitory effect of F-ara-A with other inhibitors of DNA synthesis, aphidicolin and hydroxyurea were used at the equimolar concentrations of F-ara-A in the same experimental setting (Fig. 6). By the pre-incubation with aphidicolin, the tail moment value at 4 h was high, and the thymidine incorporation was reduced, compared with the respective values for UV alone. But these effects

were less than those by F-ara-A (P = 0.001 for the tail moment value at 4 h, P = 0.01 for the radioactive count at 6 h, by the paired t test between UV + F-ara-A and UV + aphidicolin). Hydroxyurea showed no effects on the tail moment and thymidine uptake (P = 0.35 for the tail moment value at 4 h, P = 0.4 for the radioactive count at 6 h, by the paired t test between UV alone and UV + hydroxyurea).

Enhancement of UV-induced cytotoxicity by F-ara-A. To evaluate the subsequent effect on cellular viability, apoptotic cell death was determined at 24 h after lymphocytes were treated with F-ara-A, or with UV, or with both in combination. The combination provided more than additive apoptotic cell death than the sum of each treatment alone (Fig. 7).

DISUCSSION

UV induces cyclobutane primidine dimers and photo adducts, which are exclusively repaired by nucleotide excision repair.[13] The process of nucleotide excision repair consists of recognition of the damaged DNA, dual incision and excision of the oligonucleotides including the damaged nucleotide, gap filling by the

resynthesis of DNA, and rejoining by ligation.[10-13] For the evaluation of the repair kinetics, the Comet assay was used because the initial step of the incision and the last step of the rejoining were measurable as the amount of DNA strand breaks by this method. As the DNA resynthesis step was measured by the incorporation of tritiated thymidine into DNA, the major steps of the repair were evaluable by these two techniques.

Here, we demonstrated that normal lymphocytes were able to initiate nucleotide excision repair after UV exposure. The significant increase in the tail moment value that was observed at 1 h after the exposure suggests the maximal incision of the repair process (Figs. 1A and 3A). The subsequent gap filling by DNA resynthesis was demonstrated by the incorporation of tritiated thymidine into DNA. The uptake of thymidine reached a plateau at 4 h, suggesting the completion of this process (Fig. 4). The result was almost compatible with those that previous studies have demonstrated.[21, 25] This behavior might be associated with the decrease in the tail-moment values in later times. After the initial increase, the tail moment declined with time (Fig. 1A). This diminution of the tail-moment values represents the disappearance of the DNA single strand breaks that had been incised in the initial

step of the repair process. Therefore, the tail moment back to the control level at 4 h would represent the completion of the repair by successful rejoining of the incised DNA (Figs. 1A and 3B).

We previously demonstrated that when normal lymphocytes were stimulated to undergo repair by a 30-min incubation with a prodrug of cyclophosphamide, 4-hydroperoxycyclophosphamide (4-HC), the repair started at least at the end of the incubation period and completed its process within 4 h.[30] Our present study showed the increase in the tail moment value at 30 min after UV, suggesting that the repair process had already begun. Thus, these results indicate that in such experimental settings the repair response is very prompt in the onset and rapid for completion of its process.

Pretreatment of F-ara-A inhibited UV-induced repair in a concentration-dependent manner (Fig. 5). This effect was demonstrated by the inhibition of thymidine incorporation and the retention of the high tail moment value at 4 h. These results suggest that F-ara-A inhibits mainly the DNA resynthesis step and the rejoining step of the repair process. These observations would be compatible with the essential activity of F-ara-A being a DNA chain terminator and an

inhibitor of ligation.[16-19)]

The previous study demonstrated that UV-induced nucleotide excision repair in normal lymphocytes permitted an incorporation of F-ara-AMP into the repair patch when the cells were pre-treated with 10 μM F-ara-A.[21)] This increased incorporation of F-ara-A was closely correlated to the inhibition of thymidine incorporation into DNA during the repair, suggesting the inhibition of DNA resynthesis. Because F-ara-A at this concentration (10 μM) was minimally toxic to normal lymphocytes, the inhibition of the thymidine uptake was not due to the acute cytotoxixicity of F-ara-A.[21)] Thus, the inhibition of the repair demonstrated in the present study could be specifically attributed to the mechanistic interaction between the UV-induced repair and F-ara-A, not to the simply reduced viability by the immediate cytotoxicity of F-ara-A.

Moreover, it was previously demonstrated that a pre-incubation of F-ara-A before the addition of 4-HC was more inhibitory to the repair process than a post-incubation of F-ara-A after 4-HC.[30)] This suggests that the period during which the repair is susceptible to inhibitors is very short. This would be because of the prompt and rapid action of the repair. Therefore, inhibitors of the repair should be present prior to the initiation of the repair for maximizing the inhibition. Thus, we

used F-ara-A as pre-treatment.

F-ara-A, aphydicolin, and hydroxyurea are inhibitors of DNA synthesis in S phase, which should substantially be non-toxic to non-cycling normal lymphocytes. However, in the present study, these drugs were used prior to the initiation of the repair by UV exposure. We have chosen this low concentration (5μM) for these drugs to avoid a minimal chance of immediate loss of cellular activity including DNA repair capacity by these agents, because we investigated how inhibitory these drugs were mechanistically to the repair process. Thus, the concentration of these agents was equimolar and equally non-toxic (5μM).

Aphidicolin, a DNA polymerase inhibitor, showed some inhibition of the repair (Fig. 6). But this drug was less effective with regard to the inhibition of the thymidine uptake and the retention of the comet tail at 4 h, compared with F-ara-A. This might be due to the multifactorial actions of F-ara-A, i.e. the inhibition of DNA polymerase, the termination of DNA chain elongation, the inhibition of ligase, and the inhibition of ribonucleotide reductase.[16-19, 26, 31] Hydroxyurea, a ribonucleotide reductase inhibitor, exhibited no inhibition on the repair process. The concentration of hydroxyurea used here might not have been higher enough to reduce the nucleotide

pool.[27, 32] Thus, unlike aphidicolin nor hydroxyurea, F-ara-A with agents evoking a DNA repair response may have more profound effects on cell viability (Fig. 7). In addition, the inhibition by these agents would not be attributed to the residual prolonged effects because of the prompt and rapid action of the repair.

In conclusion, the present study clearly demonstrated that F-ara-A inhibited nucleotide excision repair in normal lymphocytes initiated by UV. This suggests that nucleoside analogues would also inhibit the excision repair initiated by alkylating agents or platinum analgues in the same manner. Such modulation may enhance cytotoxicity because the DNA damage induced by these agents can remain in the DNA and/or the nucleoside analogues incorporated into DNA can exert their original cytotoxicities (Fig. 7).[30, 33] In this sense, DNA repair may be considered as a biologic target for a chemotherapeutic strategy against cancer. Moreover, resistant cells with an increased repair capacity may be more sensitive to such a strategy if the repair function facilitates the incorporation of the analogues into DNA.

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Legends

Fig. 1 Normal lymphocytes from 6 healthy donors were exposed to UV, and tail moments were determined at 0 h, 0.5 h, 1 h, 2 h, 4 h after the irradiation. (A) The kinetics of nucleotide excision repair initiated by 3 J/m^2 UV (mean ± SD). (B) Dose-dependent response (tail moments at 1 h) to UV exposure (mean ± SD). The tail-moment value of untreated cells was set as the control (hair line).

Fig. 2 Effect of F-ara-A on UV-induced DNA repair. Normal lymphocytes from 6 healthy donors were pre-incubated for 2 h with F-ara-A at 0.1 or 10 μM, followed by 3 J/m^2 UV exposure and by immediate washing the cells into fresh media. Tail moments were determined at 0 h, 0.5 h, 1 h, 2 h, and 4 h. (•); F-ara-A 10 μM, (o); F-ara-A 0.1 μM, (mean ± SD). The tail-moment value of untreated cells was set as the control (hair line).

Fig. 3 The Comet formation. Normal lymphocytes were pre-incubated for 2 h with or without 10 μM F-ara-A, followed by 3 J/m^2 UV exposure. After immediate washing the cells into fresh media, the samples were applied to the Comet assay at 1 h and 4 h. The comet tail produced by UV at 1 h (A) almost disappeared at 4 h (B). In contrast, the comet tail generated by the combination treatment of UV with F-ara-A

(C) remained even at 4 h (D). (A) UV alone at 1 h, (B) UV alone at 4 h, (C) F-ara-A & UV at 1 h, (D) F-ara-A & UV at 4 h.

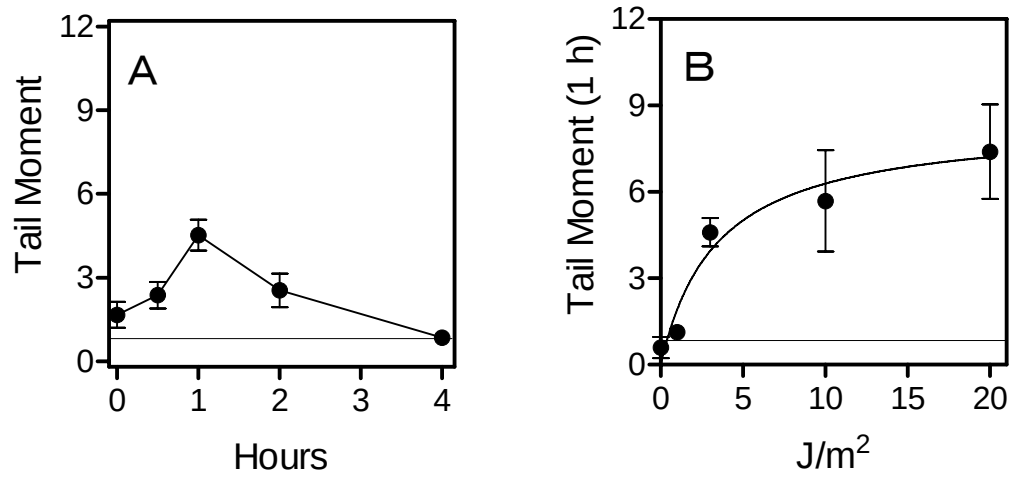
Fig. 4. The effect of F-ara-A on DNA resynthesis. Normal lymphocytes from 3 healthy donors were pre-incubated for 2 h with F-ara-A at 10 μM (•), or not (o), followed by 3 J/m^2 UV exposure. After immediate washing into fresh media, the samples were incubated with tritiated thymidine for 0 h, 1 h, 2 h, 4 h, and 6 h, and the radioactive counts were determined (mean ± SD).

Fig. 5. Concentration-dependent inhibition of DNA repair by F-ara-A. Normal lymphocytes from 6 healthy donors were pre-incubated for 2 h with the escalating concentrations (0.001, 0.01, 0.1, 1, 5, and 10 μM) of F-ara-A or not, followed by 3 J/m^2 UV exposure and by immediate washing the cells into fresh media. Tail moments were determined at 4 h after the drug washout (mean ± SD) (A), whereas the radioactivities of DNA were measured at 6 h after the subsequent incubation with tritiated thymidine following the drug washout (mean ± SD) (B). The rate of DNA resynthesis was defined as the relative value (%) of the radioactivity for the combination treatment to that for UV alone in (B).

Fig. 6. Comparison of the inhibitory effect among aphidicolin, hydroxyurea, and

F-ara-A. Normal lymphocytes from 3 healthy donors were preincubated for 2 h with 5 μM F-ara-A (o), or 5 μM aphydicholin (Δ), or 5 μM hydroxyurea (□), or not (●), followed by 3 J/m^2 UV exposure and by immediate washing the cells into fresh media. The lymphocytes were incubated and applied to the comet assay at 0 h, 1 h, 2 h, and 4 h (mean ± SD) (A), or incubated with tritiated thymidine for 6 h to determine the radioactivity of DNA (mean ± SD) (B). The tail-moment value of untreated cells was set as the control (hair line) in (A). The rate of DNA resynthesis was determined as the relative value (%) of the radioactivity for each combination treatment to that for UV alone in (B). AP; aphidicolin. HU; hydroxyurea.

Fig. 7 Enhancement of UV-induced cytotoxicity by F-ara-A. Normal lymphocytes from 3 healthy donors were treated with a 2-h incubation with 10 μM F-ara-A, or 3 J/m^2 UV exposure, or a 2-h pre-incubation with 10 μM F-ara-A followed by 3 J/m^2 UV exposure. At 24 h after the treatments, the apoptotic cell death was measured by the nuclear morphology using Hoechst No. 33342 staining (mean ± SD).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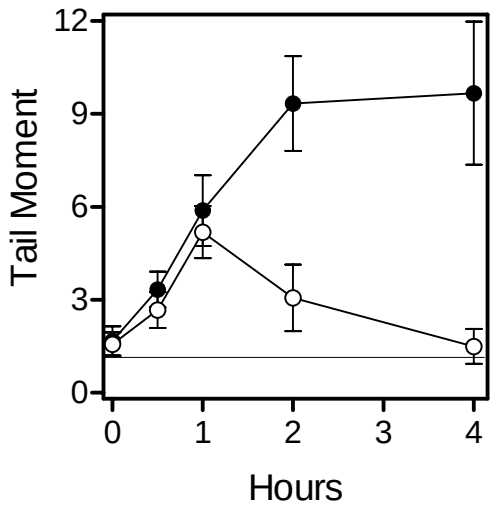
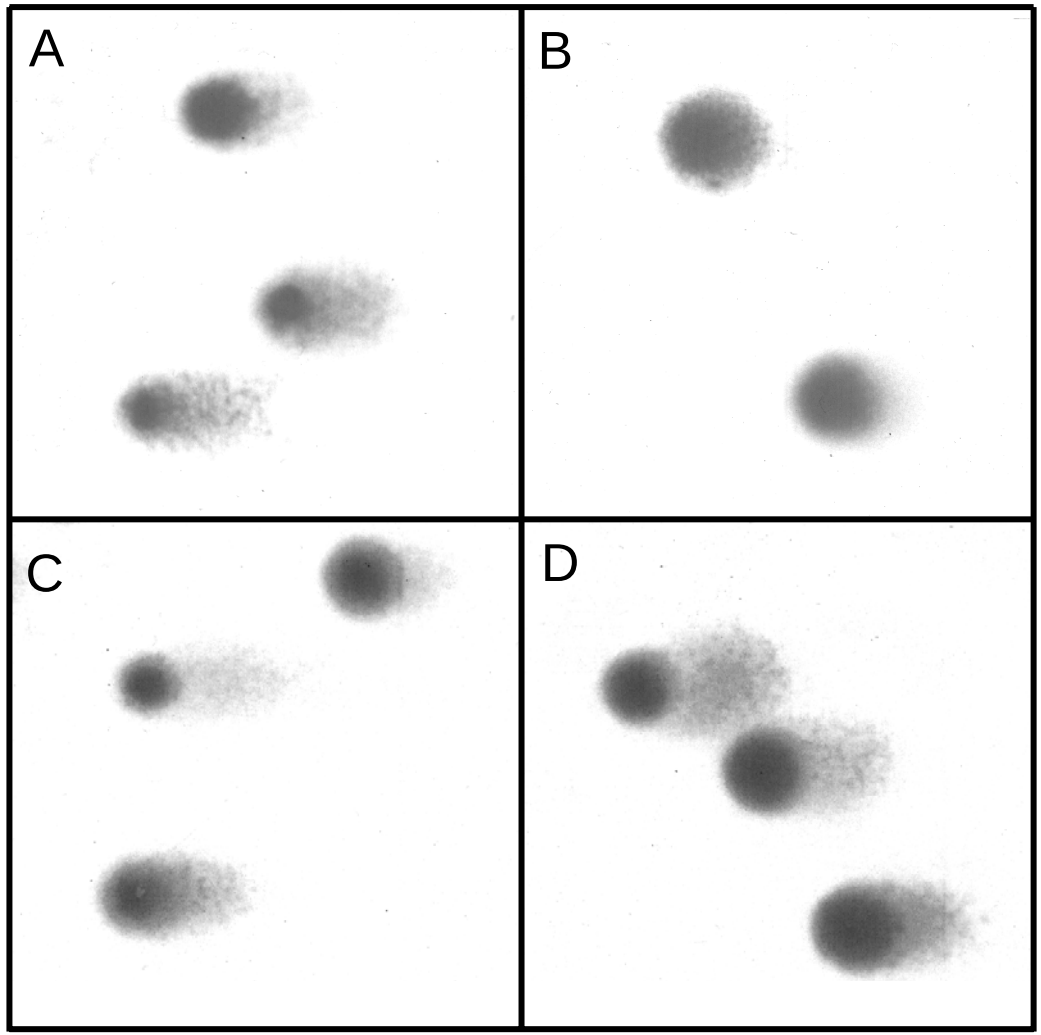
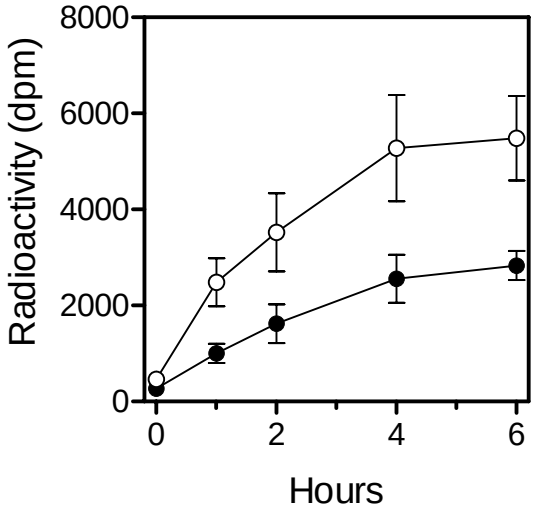
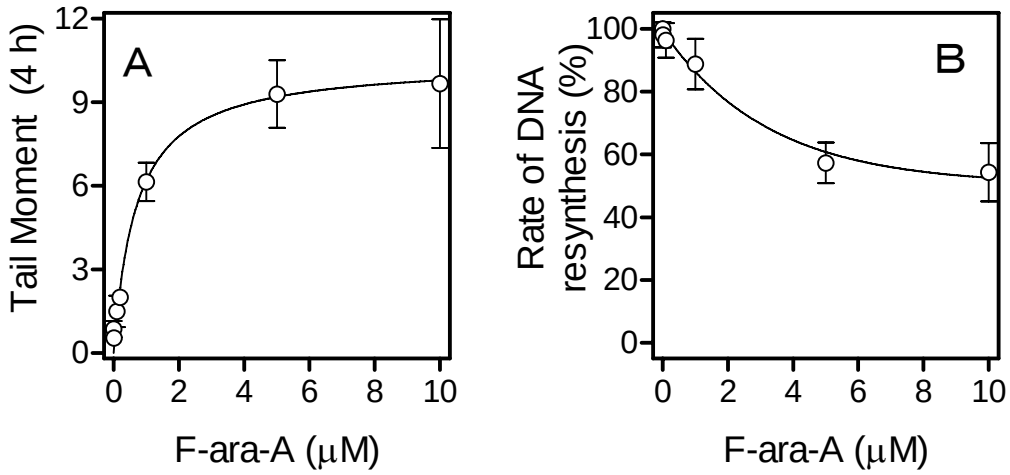
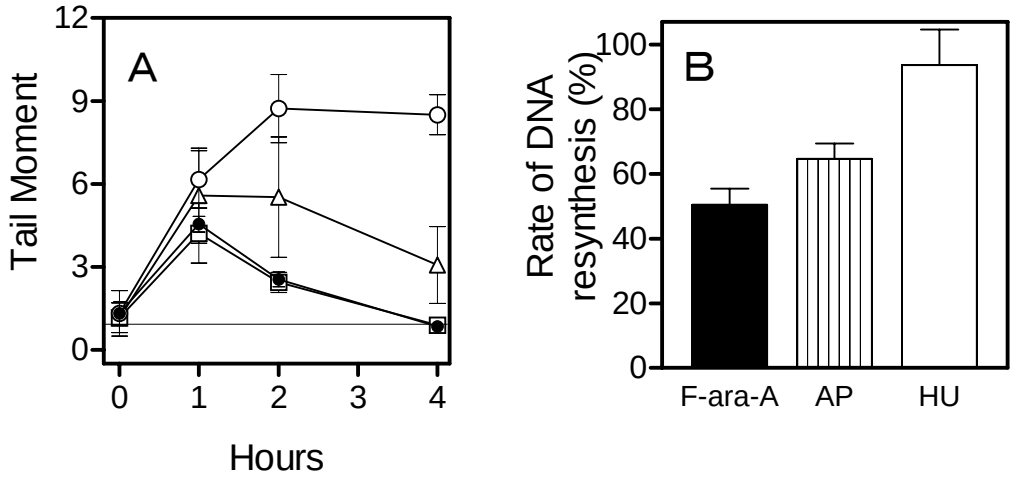
20
J/m2

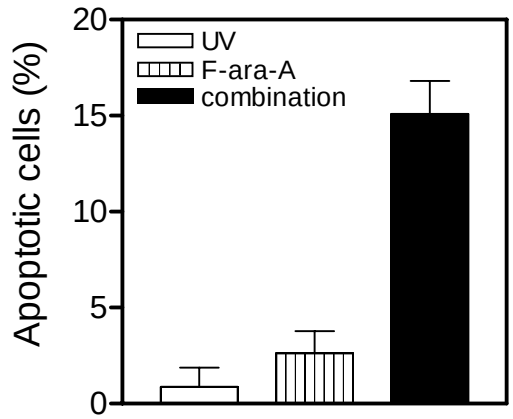

12
9
6
3
0
Tail Moment
0
1
2
3
4
Hours


A
B
C
D


8000
6000
4000
2000
0
Radioactivity (dpm)
0
2
4
6
Hours


A
Tail Moment (4 h)
12
9
6
3
0
0
2
4
6
8
10
F-ara-A (μM)
B
Rate of DNA resynthesis (%)
100
80
60
40
20
0
0
2
4
6
8
10
F-ara-A (μM)


A
Tail Moment
12
9
6
3
0
0
1
2
3
4
Hours
B
Rate of DNA resynthesis (%)
100
80
60
40
20
0
F-ara-A
AP
HU


Apoptotic cells (%)
20
15
10
5
0
UV
F-ara-A
combination